REMARKS

Claims 108, 110-114 and 116-119 are currently pending in the application. The Examiner has withdrawn from consideration claims 112 and 113, which were allowed per Paper No.: 9 (Office Action issued January 15, 2003). It is Applicant's understanding that claims 112 and 113 will be reinstated upon allowance of the claims currently under consideration.

Claims 108, 110, 111, 114 and 116-119 are under consideration and stand rejected under 35 U.S.C. § 103(a) based on a number of positions laid out in detail in the 10/06/04 Final Office Action. Claims 110 and 116 are rejected under 35 U.S.C. § 102(b) over the Nudelman and Windmuller references cited in a previous office action.

Applicant is submitting herewith additional arguments in support of their rebuttal of the Examiner's 103(a) rejection in their 1/6/05 response.

In her 10/06/04 Final Office Action, the Examiner states that Kaizu and Nudelman provide the motivation to one skilled in the art to make antibodies to the claimed epitope because:

(i) Kaizu *et al.* teach that the KH1 antibody, which binds to the disclosed epitope, shows higher specificity for human colon adenocarcinoma; and

(ii) Nudelman *et al.* teach that the claimed epitope is a major component of Le^y-active components detected in human colonic carcinoma cases, that the expression of the Le^y antigen has diagnostic and prognostic value, and that the addition of the third fucosyl group provides antigenicity for the glycolipid, citing Kaizu *et al.*

Applicant points out that in both Kaizu's and Nudelman's reports, the Le^y glycolipids (including the trifucosylnonaosylceramide) are *naturally occurring* entities isolated from human cancer cells. Applicant submits that, while Kaizu *et al.* teach that the KH1 antibody showed higher specificity for Le^y trifucosylnonaosylceramide (*natural* antigen), the skilled practitioner would not have been motivated to make the claimed epitope for use as immunogen, in light of the teachings of Kitamura *et al.* (Proc. Natl. Acad. Sci. USA, 1994, 91:12957-12961). In that

paper, Kitamura *et al.* teach that synthetic Le^y generates antibodies in mice, but that the antibodies generated react only with the synthetic antigen used for vaccination and not with natural Le^y. See page 12960, paragraph bridging columns 1 and 2:

> "However, several key features distinguished antibodies raised against synthetic Le^y determinants and those raised against natural Le^y. (i) The anti-synthetic Le^y mAbs reacted well against synthetic Le^y determinants but poorly against natural Le^y; (ii) the isotypes of the antibodies raised against synthetic Le^y were IgG1, IgG2a, or IgG2b, in contrast to the IgM or IgG3 isotypes of antibodies to natural Le^y; and (iii) even the most specific of the anti-synthetic Le^y mAbs cross-reacted with Le^x of H-type 2 structures, whereas anti-natural Le^y mAbs could be isolated that showed apparent exclusive specificity for Le^y. ... We have made attempts to modify the immune response to synthetic Le^y using different adjuvants and different immunization procedures, but these have not succeeded in changing specificity or mAb isotypes". (Emphasis added)

Kitamura *et al.* teach that one *cannot* use synthetic Le^y with any expectation of success, much less a reasonable expectation of success, because the antibodies generated by synthetic Le^y do not bind to natural Le^y, are of different types, and are non-specific.

Therefore, the skilled practitioner, in light of the teachings of Kitamura *et al.*, would not be reasonably motivated to synthesize the structurally related Le^y trifucosylnonaosyl determinant disclosed by Kaizu and Nudelman, because there is no reasonable expectation of success that the *synthetic* antigen could be useful in immunotherapy. Specifically, there is no reasonable expectation of success that the claimed *synthetic* Le^y trifucosylnonaosyl determinant would be useful to elicit antibodies in a subject which are specific for and actually bind to Le^y trifucosylnonaosyl expressing epithelial tumor cells, *i.e.*, to *natural* Le^y trifucosylnonaosyl. In fact, based on the teachings of Kitamura *et al.*, a person of ordinary skill in the art would likely question whether *synthetic* Le^y trifucosylnonaosyl may be useful in immunotherapy. Applicant asserts that, at the time of the invention, in light of the teachings of Kitamura *et al.*, one of

ordinary skill in the art could not reasonably find motivation from Kaizu and Nudelman's disclosure of the *natural* Le^y trifucosylnonaosyl to prepare the claimed synthetic constructs, because there was no reasonable expectation that they would have the requisite immunogenic properties.

To further illustrate the lack of reasonable expectation of success in the art, Applicant points, for example, to the teachings of Livingston *et al.* "Antibodies against GD2 ganglioside can eradicate syngeneic cancer micrometastases" *Cancer Research*, 1998, 58:2844-2849. In that report, Livingston notes that "GM2-KLH and GD2-KLH have both proven consistently immunogenic and safe in melanoma patients, whereas GD3 (the major melanoma ganglioside)-KLH has not proven so immunogenic" – See column 1, first sentence, page 2848. As taught by Livingston *et al.*, two constructs (such as, for example, GM2 and GD3) can have drastically different immunogenic properties even though they are structurally closely related. In other words, no construct can be presumed to work even if a structurally related construct has succeeded. The Livingston paper serves to illustrate that, even after the invention was made, one could not reasonably predict the immunogenicity of carbohydrate antigens without first conducting relevant testing. Applicant asserts that experimentation is required to assess the utility of tumor-associated carbohydrate antigens in immunotherapy.

Based on the teachings of Kitamura *et al.* alone, Applicant respectfully submits that the Kaizu and Nudelman references of record could not reasonably provide motivation to the skilled artisan at the time the invention was made to make the claimed constructs. Specifically, the teachings of Kaizu and/or Nudelman are not sufficient to provide motivation to make the claimed Le^y trifucosylnonaosyl constructs. Based on the knowledge available in the art at the time the invention was made (*e.g.*, Kitamura *et al.*), there could not be, in fact, any expectation of success without experimental testing of the claimed synthetic Le^y trifucosylnonaosyl constructs. Applicant therefore maintains that there is no motivation in the combination of the cited

references of record, and no expectation of success in the combination. Accordingly, the Examiner's rejection under § 103(a) is improper because at least one of the requirements for establishing a *prima facie* case of obviousness is lacking (*e.g.*, reasonable expectation of success). Applicant respectfully requests that the stated 103 rejection be withdrawn.

CONCLUSION

Applicant thanks the Examiner for his/her time and consideration. If a telephone conversation would help clarify any issues, or help expedite prosecution of this case, Applicant invites the Examiner to contact the undersigned at (617) 248-5150.

It is not believed that fees are required, beyond those which may otherwise be provided for in documents accompanying this paper. However, in the event that any additional fees required for consideration of this paper, such fees are authorized to be charged to our Deposit Account No. 03-1721.

Respectfully submitted,

Date: February 7, 2005

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Immunology

Specificity analysis of blood group Lewis-y (Le^y) antibodies generated against synthetic and natural Le^y determinants

(anti-Le^y antibody/humanized antibody/synthetic neoglycoprotein)

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Contributed by Lloyd J. Old, July 11, 1994

ABSTRACT **Le^y-reactive monoclonal antibodies (mAbs) were generated in mice by immunization with synthetic Le^y neoglycoproteins or with Le^y-expressing cells. Serological analysis indicated that mAbs raised against synthetic Le^y (*i*) reacted strongly with synthetic Le^y but poorly with natural Le^y, (*ii*) cross-reacted with Le^x or H-type 2 structures, and (*iii*) were IgG1, IgG2a, or IgG2b. mAbs raised against Le^y-expressing cells (*i*) reacted with both synthetic Le^y and natural Le^y, (*ii*) were of two types: cross-reactive with Le^x or H-type 2 structures or specific for Le^y, and (*iii*) were IgM or IgG3. One of the mAbs raised against natural Le^y, mAb 3S193 (IgG3), showed high specificity for Le^y in ELISA tests with synthetic Le^y and Le^y containing glycoproteins and glycolipids; it also reacted strongly in rosetting assays and cytotoxic tests with Le^y-expressing cells. mAb 3S193 did not lyse O, A, AB, and B human erythrocytes in the presence of human complement. In flow cytometry, there was weak reactivity with granulocytes, a reactivity also observed with two previously described highly specific Le^y mouse mAbs—BR55-2 (IgG3) and B3 (IgG1). A humanized version of mAb 3S193 has been constructed, and the specificity pattern and reactivity for Le^y remain very similar to mouse mAb 3S193.**

The serological analysis of human cancer cells with mouse monoclonal antibodies (mAbs) has identified a number of carbohydrate determinants, linked either to lipids (glycolipids) or to proteins (glycoproteins) (1–5). Blood group-related (BGR) antigens—mainly, the lacto- (type 1) and neolacto- (type 2) structures—have been the focus of much attention because of their strong expression on tumors of epithelial origin (1–5). Although BGR antigens are also expressed in normal tissues, there is evidence for altered expression in certain tumor types (1–6). We and others showed that Le^y {Fuc(α1 → 2)Gal(β1-4)[Fuc(α1 → 3)]GlcNAc} antigen accumulates to a higher level in colonic cancer than in the adjacent normal colon epithelium (7, 8). Because Le^y is expressed in >70% of epithelial cancers, such as breast, ovary, colon, and lung cancer, there is considerable interest in its use as a target for mAb imaging and therapy. A large number of Le^y-reactive mAbs have been generated and subjected to various degrees of specificity analysis, particularly in relation to their reactivity with Le^x {Gal(β1 → 4)[Fuc(α1 → 3)]GlcNAc} and H-type 2 [Fuc(α1 → 2)Gal(β1 → 4)GlcNAc] structures, two determinants structurally related to Le^y (9–16). The importance of a detailed serological analysis before an anti-Le^y reagent is used in humans is illustrated by our findings with a number of Le^y antibodies originally thought to be specific for Le^y but later shown to cross-react with Le^y-related structures—especially Le^x or H-type 2—and to agglutinate human erythrocytes (14). For this reason, we initiated an effort to generate Le^y reagents with improved reactivity and specificity. The availability of a wide range of synthetic carbohydrate structures (17) greatly facilitates the specificity testing of mAbs to Le^y and other BGR antigens. In addition, synthetic Le^y used as an immunogen offers opportunities for generating anti-Le^y reagents. In the present study, we have analyzed in detail the specificity of newly derived mAbs raised against synthetic or natural Le^y. One of the most specific and reactive Le^y mAbs derived in this series, mAb 3S193, has been humanized, and the reactivities of the humanized mAb 3S193 and mouse mAb 3S193 are compared.

MATERIAL AND METHODS

Tissue Culture and mAb. Tumor cell lines were obtained from the tumor cell bank in the Ludwig Unit at Memorial Sloan–Kettering Cancer Center and maintained as described (18). mAbs 118 and F-12 have been described (14, 15). mAbs BR55-2 (IgG3) (12) and B3 (IgG1) (16) were provided by Z. Steplewski (Wistar Institute, Philadelphia) and I. Pastan (National Institutes of Health, Bethesda, MD), respectively.

Synthetic Neoglycoproteins. Le^y-human serum albumin (HSA), Le^y-keyhole limpet hemacyanin (KLH), H-type 2-bovine serum albumin (BSA), Le^x-BSA, lacto-*N*-neotetraose (LNneoT)-BSA, A type 1-BSA, ALe^b-BSA, B type 1-BSA, B type 2-BSA, and BLe^b-BSA were obtained from Chembiomed (Edmonton, Canada). Le^y-BSA, Le^b-BSA, H-type 1-BSA, and Le^a-BSA were purchased from BioCarb (Lund, Sweden).

Blood Group Glycoproteins and Glycolipids. Blood group-active glycoproteins—i.e., A(MSS), B(Beach), Le^y/Le^b (Tighe), and Le^x/Le^a (N-1) from ovarian cyst fluids and hog gastric A+H mucin—and purified standard glycolipids—i.e., H-type 1, H-type 2, Le^a, Le^b, Le^x, and Le^y were prepared as described (14, 19).

Immunizations. mAbs were produced by using the standard hybridoma technique after four to six immunizations of BALB/c mice with 2.5–50 μg of Le^y-HSA or 3 to 10 × 10^6 of MCF-7 breast or HCT-15 colon adenocarcinoma cells. Culture supernatants from hybridomas were screened by mixed hemadsorption (MHA) rosetting assays and cytotoxic tests with tumor cells and ELISA with synthetic Le^y neo-

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Abbreviations: mAb, monoclonal antibody; ADCC, antibody-dependent cellular cytotoxicity; MHA, mixed hemadsorption; BGR, blood group-related; BSA, bovine serum albumin; KLH, keyhole limpet hemacyanin; HSA, human serum albumin.

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glycoprotein. The immunoglobulin subclass of mAbs was determined by the Ouchterlony immunodiffusion method.

Serological Assays. *MHA assays.* Protein A-MHA and rabbit anti-mouse immunoglobulin-MHA assays for the detection of cell surface antigens were done as described (18, 20).

Cytotoxic tests for complement-dependent cytotoxicity. Ten microliters of a cell suspension (1.5×10^4/ml) was distributed into wells of microtiter plates (Nunclon; Nunc) and incubated for 20 hr at 37°C in 5% CO_2/95% air. The medium was removed, 10 μl of serially diluted antibody was added to each well, and incubation was for 45 min. Then 10 μl of complement (human serum diluted 1:3) was added. Tests were done in duplicate with medium, antibody, and complement controls. After 4 hr, plates were fixed with methanol for 10 min, rinsed in distilled water, stained with 2% Giemsa stain in phosphate-buffered saline for 25 min, and rinsed. Plates were analyzed under the light microscope, and the percentage cytotoxicity of a given antibody dilution was calculated as follows: [1 − (number of cells in well treated with antibody and complement/number of cells in well treated with medium only)] × 100.

ELISA. ELISA was done with natural glycoproteins and glycolipids and synthetic neoglycoproteins adsorbed to the wells of microtiter plates as described (21).

Tests for antibody-dependent cellular cytotoxicity (ADCC). A short-term ^{51}Cr-release test was done with some modification (22). Target cells (10^6) were labeled with 100 μCi (3.7 MBq; 1 Ci = 37 GBq) of ^{51}Cr for 1 hr at 37°C; labeled cells were seeded (10^4 cells per well in 50 μl) into 96-well flat-bottom plates and incubated for 2 hr. Then, antibody (50 μl per well) and human lymphocytes (50 μl per well) were added. After incubation for 6 hr at 37°C, supernatants were harvested, and radioactivity was measured with a γ counter. Spontaneous release was defined as the cpm released in medium alone instead of in mAb and lymphocytes, and maximum release was defined as the cpm released by Nonidet P-40. Percentage cytotoxicity was calculated as follows: [(sample release − spontaneous release)/(maximum release − spontaneous release)] × 100. Spontaneous release was <35% of maximum release in all experiments.

Hemolysis test. The ^{51}Cr-release assay also was used to test hemolysis by mAbs. Briefly, 0.1% ^{51}Cr-labeled erythrocytes were incubated with purified mAb and autologous complement for 30 min, and the radioactivity in the supernatants was measured. Percentage lysis was calculated according to the same formula used in ADCC assays. Spontaneous release was <1% of maximum release in all experiments.

Flow Cytometry Analysis (Peripheral Blood Leukocyte Assay). The reactivity with peripheral blood leukocytes was analyzed with purified mAbs using a FACStar flow cytometer (Becton Dickinson) as described (14).

Immunohistochemical Procedures. Tissues were obtained through the Tumor Procurement Service of the Department of Pathology, Memorial Hospital, embedded in OCT compound (Miles), snap-frozen in isopentane precooled in liquid N_2, and stored at −70°C. Sections (5 μm thick) were cut, mounted on poly(L-lysine)-coated slides, air-dried, and fixed in acetone (4°C, 10 min). mAbs were used at 0.5–20 μg/ml, and the avidin–biotin immunoperoxidase procedure was done as described (23).

Generation of Humanized and Chimeric Antibodies. mAb 3S193 was humanized by the principles of Riechmann *et al.* (24). The humanized heavy-chain variable regions contained the complementarity-determining regions of mAb 3S193 and the framework regions of the human KOL heavy chain, incorporating one (HuVH) or two (HuVHT) mouse framework residues at positions 28 and 24/28, respectively [numbering according to Kabat *et al.* (25)]. The humanized κ chain was based on the human REI κ-chain frameworks and included no mouse framework residues (HuVK) or a substitution at Kabat position 71 (HuVKF). DNAs encoding these domains were expressed with human IgG1 and κ constant-region genes using a vector system based on that of Orlandi *et al.* (26). Similar constructs, containing mouse mAb 3S193 variable-region DNAs, allowed synthesis of chimeric antibody chains, termed MuVH and MuVK. Antibodies containing different combinations of heavy and light chains were produced by cotransfection of myeloma cells as described (27); antibodies composed of a mixture of one chimeric and one humanized chain are hereafter referred to as "hybrid" antibodies. The antibodies described here are numbered as follows: 3S193#5, MuVH/MuVK; 3S193#6, MuVH/HuVK; 3S193#7, HuVH/HuVK; 3S193#11, HuVHT/HuVKF.

RESULTS

Generation of Mouse Anti-Le^y Antibodies. Two approaches to generate mouse anti-Le^y antibodies were compared: one using chemically synthesized Le^y neoglycoproteins as the immunogen and the other using cultured human tumor lines expressing Le^y on the cell surface. Culture supernatants from hybridomas were initially screened for (*i*) Le^y–KLH and KLH reactivity using ELISA and (*ii*) cell-surface reactivity using MHA rosetting assays and cytotoxic tests on three cell lines, MCF-7 (Le^{y+}), HCT-15 (Le^{y+}), and SK-MEL-28 (Le^{y-}). On the basis of these tests, five mAbs generated against synthetic Le^y and five mAbs generated against Le^y-expressing cells were selected for detailed serological analysis. These results are summarized in Tables 1 and 2.

Anti-synthetic Le^y mAbs. The isotypes of the anti-synthetic Le^y mAbs were IgG1, IgG2a, or IgG2b. These mAbs showed strong and equal reactivity with Le^y conjugated to HSA, BSA, or KLH and no reactivity with a number of other synthetic oligosaccharides conjugated to BSA with the same linker, excluding a significant contribution of carrier protein or linker moiety. However, none of the five mAbs were specific for Le^y; four of them strongly cross-reacted with Le^x, and one cross-reacted with H-type 2 determinants. The anti-synthetic Le^y mAbs did not react with Le^y containing natural glycoproteins or glycolipids in ELISA and were poorly reactive with cells expressing Le^y in rosetting and cytotoxic assays.

Anti-natural Le^y mAbs. The isotypes of the anti-natural Le^y mAbs were IgM or IgG3. Their reactivity with synthetic Le^y was weaker than mAbs to synthetic Le^y, but they reacted strongly with Le^y-containing natural glycolipids and glycoproteins and showed 2- to 4-logarithmic-higher titers in assays using Le^y-expressing cells. As with anti-synthetic Le^y mAbs, the main cross-reactions of anti-natural Le^y mAbs were with Le^x and with H-type 2 structures. However, two mAbs in this series, 3S193 and 8S202, showed a high degree of specificity and reactivity for Le^y determinants. In hemolysis tests, mAb 8S202 was strongly hemolytic for O erythrocytes and to a lesser degree for A, AB, and B erythrocytes. In contrast, mAb 3S193 did not lyse erythrocytes. This strong hemolytic activity of mAb 8S202 cannot be accounted for by its weak cross-reactivity with H-type 2 (at a mAb concentration of 100 μg/ml) because mAb 118 (see below), which is less hemolytic, has a stronger cross-reactivity with H-type 2 (6.25 μg/ml). In flow cytometry, mAb 8S202 showed no reactivity with granulocytes, whereas mAb 3S193 showed low granulocyte reactivity (mean positive count, 19.7). This level of reactivity is >10-fold lower than the reactivity of anti-Le^x mAb P12 on granulocytes (mean positive count, 267) in fluorescence intensity.

Immunohistochemical Staining. The reactivity of mAbs with normal and malignant tissues was examined. Of three

Table 1. Specificity of mAbs with synthetic neoglycoproteins, glycoproteins, and glycolipids

	Synthetic antigen*								Glycoprotein†					Glycolipid†			
Antibody	Le^y	H-2	Le^x	LNneoT	H-1	Le^a, Le^b	ALe^b, BLe^b	A-1, B-1, B-2	A	B	Tighe	N-1	Hog	Le^y	H-2	Le^x	H-1, Le^a, Le^b
Anti-synthetic Le^y																	
2A1 (IgG2b)	0.025	100	0.025	−	±	−	−	−	−	−	∓	−	−	−	−	−	−
2A4 (IgG2b)	0.025	±	0.10	−	−	−	−	−	NT	NT	NT	NT	NT	NT	NT	NT	NT
2A7 (IgG2a)	0.025	±	0.10	−	−	−	−	−	−	−	−	−	−	−	−	−	−
2A19 (IgG1)	0.025	100	0.10	100	±	±	−	−	NT	NT	NT	NT	NT	NT	NT	NT	NT
2A37 (IgG2b)	0.39	1.56	±	±	±	±	±	±	−	−	−	−	−	−	−	−	−
Anti-natural Le^y																	
3A5 (IgG3)	0.10	−	6.25	−	−	−	−	−	−	−	++	−	−	++	−	++	−
3S193 (IgG3)	0.10	−	100	−	−	−	−	−	−	−	++	−	−	++	−	±	−
3S209 (IgM)	0.10	100	25	100	100	100	100	100	−	+	++	∓	∓	++	−	++	−
8S202 (IgG3)	0.39	100	−	−	−	−	−	−	−	∓	+	−	−	+	−	−	−
8A15 (IgG3)	0.10	25	−	−	−	−	−	−	−	+	++	−	∓	++	−	−	−
Previously described anti-Le^y																	
BR55-2 (IgG3)	0.10	−	100	−	−	−	−	−	−	−	++	−	−	++	−	−	−
B3 (IgG1)	0.10	−	25	−	−	−	−	−	−	−	++	−	−	++	−	−	−
118 (IgG3)	0.39	6.25	25	25	25	25	25	25	−	±	++	−	±	++	+	∓	−
F12 (IgM)	0.39	±	±	±	±	±	±	−	−	∓	++	−	−	NT	NT	NT	NT
Engineered 3S193																	
Chimeric #5	0.10	50	100	−	−	−	−	−	−	−	++	−	∓	NT	NT	NT	NT
Hybrid #6	0.025	6.25	25	100	100	±	±	−	−	∓	++	−	∓	NT	NT	NT	NT
Humanized #7	1.56	−	±	−	−	−	−	−	−	−	++	−	−	NT	NT	NT	NT
Humanized #11	0.39	±	±	−	−	−	−	−	−	−	++	−	−	NT	NT	NT	NT

*Values are minimum antibody concentrations (μg/ml) that show OD >0.6 in ELISA. Synthetic oligosaccharides conjugated to BSA were used as antigens. In addition, Le^y-HSA and Le^y-KLH were also tested and gave the same results as Le^y-BSA. Each mAb was titrated from 100 μg/ml. −, Negative; ±, weakly positive at highest concentration tested (100 μg/ml).

†Antibody concentration of 5 or 10 μg/ml was used for extracted antigens. −, Negative; ∓, trace; ±, weakly positive; +, positive; ++, strongly positive. NT, not tested.

mAbs generated with synthetic Le^y, mAbs 2A1 and 2A37 showed weak reactivity with some normal and malignant epithelial tissues, and mAb 2A7 was completely unreactive. By contrast, the mAbs generated with natural Le^y reacted strongly with a high proportion of carcinomas and several normal tissues. mAb 3S193 was selected for detailed analysis and stained tumor cells in 142 of the 192 human epithelial tumors tested, including tumors of the colon, stomach, breast, lung, prostate, bladder, and pancreas. Among normal tissues, prominent immunostaining with mAb 3S193 was found in the gastrointestinal mucosa and, with a heterogeneous pattern, in several other histologic types of epithelia, including breast, bronchus, pancreas, and genitourinary system. Two tissues with strong Le^x expression—namely, normal brain and spleen (28)—were also tested with mAbs 3S193 and 3A5. Both mAbs were unreactive with brain tissue. They showed no staining with spleen tissue when tested at 0.5–1 μg/ml, the concentration used for all other tissues, and staining limited to a subset of perifollicular cells, predominantly granulocytes, when tested at 10-fold higher immunoglobulin concentration.

Comparison of mAb 3S193 with Previously Described Anti-Le^y mAbs. A detailed specificity analysis was carried out on four previously described mAbs with Le^y reactivity: BR55-2 (IgG3) (12), B3 (IgG1) (16), 118 (IgG3) (14), and F-12 (IgM) (15). mAbs BR55-2 and 3S193 showed a virtually identical pattern with synthetic oligosaccharides, natural glycoproteins and glycolipids, and granulocytes. However, in rosetting assays and cytotoxicity tests, mAb 3S193 has a 10- to 100-fold-higher reactivity than mAb BR55-2. mAb B3 also showed a high degree of specificity for Le^y. mAb B3 has been reported to react with difucosylated Le^x and trifucosylated Le^y but not with Le^x at 10 μg/ml (16). At a higher concentration of mAb B3 (25 μg/ml), we find a cross-reaction of mAb B3 with Le^x. mAb 118 reacted with H-type 2 at 6.25 μg/ml, reacted with other BGR determinants at 25 μg/ml, and lysed O erythrocytes in the presence of complement. mAb F-12 showed highly restricted Le^y reactivity but was significantly weaker in cytotoxic tests with Le^y-expressing cells.

Construction and Analysis of Humanized mAb 3S193. Because of its specificity and strong reactivity for Le^y, mAb 3S193 was selected for humanization. The reactivity patterns for one chimeric, one hybrid, and two humanized versions of mAb 3S193 are shown in Tables 1 and 2. In tests with synthetic neoglycoproteins, chimeric mAb 3S193#5 showed the same titers as mouse mAb 3S193 with synthetic Le^y, but in contrast to mouse mAb 3S193, it reacted with H-type 2 structure (50 μg/ml). Hybrid mAb 3S193#6 was 5-fold more reactive than mouse mAb 3S193 with Le^y, but its cross-reactivity with H-type 2 (6.25 μg/ml) and Le^x (25 μg/ml) increased greatly. Comparison of humanized mAbs 3S193#7 and 3S193#11 shows that their Le^y specificity is identical, but #11 has higher reactivity in ELISA, protein A–MHA assay, and cytotoxic test and lower reactivity for granulocytes than #7. Fig. 1 illustrates ADCC tests using five colon and three breast cancer cell lines in the presence of mAb 35193 11 and human effector cells. Le^y-expressing cell lines show various degrees of lysis ranging from 64% (MCF-7) to 11% (SK-CO-10) with antibody concentrations as low as 0.1 μg/ml. Lytic activity correlated with Le^y expression as tested in MHA assays. Significant lysis could still be demonstrated at a mAb concentration of 0.01 μg/ml or at effector-to-target ratio of <10:1. Le^y-negative colon cancer cell line SW1222 was not lysed by humanized mAb 3S193#11 but could be lysed by an unrelated humanized mAb. A direct comparison of humanized mAb 3S193#11 and mouse mAb 3S193 showed that the ADCC activity of the humanized antibody was 100-fold greater.

Table 2. Reactivity of mAbs in rosetting assays, cytotoxic test, hemolysis test, and flow cytometry analysis

	PA-MHA*		Rb α mIg-MHA*		Cytotoxic test*		Hemolysis test,† %				Flow cytometry analysis‡					
											Lymph.		Mono.		Granulo.	
Antibody	MCF-7	HCT-15	MCF-7	HCT-15	MCF-7	HCT-15	O	A	B	AB	MPC,	%+	MPC,	%+	MPC,	%+
Anti-synthetic Le^y																
2A1 (IgG2b)	1.56	1.56	0.39	1.56	12.5	50	1	–	–	–	0.2	9	0.4	14	6.9	93
2A4 (IgG2b)	12.5	50	6.25	12.5	25	100	NT	NT	NT	NT	NT		NT		NT	
2A7 (IgG2a)	25	100	25	25	25	100	–	–	–	–	0.1	2	0.3	14	1.0	2
2A19 (IgG1)	–	–	12.5	25	–	–	–	–	–	–	NT		NT		NT	
2A37 (IgG2b)	1.56	6.25	1.56	3.13	25	100	–	–	–	–	0.1	1	0.3	13	1.4	5
Anti-natural Le^y																
3A5 (IgG3)	0.0016	0.0016	0.0016	0.0125	0.78	1.56	1	–	–	–	0.2	6	0.3	15	11.5	91
3S193 (IgG3)	0.0016	0.0016	0.0016	0.0063	0.10	0.20	–	–	–	–	0.2	11	0.3	18	19.7	99
3S209 (IgM)	–	–	0.0063	0.025	0.10	0.10	–	–	–	–	0.3	27	0.4	30	25.3	99
8S202 (IgG3)	0.0063	0.0125	0.0063	0.10	0.39	0.39	100	37	22	28	0.3	30	0.4	32	0.9	6
8A15 (IgG3)	0.0063	0.025	0.0063	0.10	1.56	1.56	79	28	NT	11	0.3	19	0.4	34	0.8	6
Previously described anti-Le^y																
BR55-2 (IgG3)	0.10	0.10	0.10	0.39	0.78	1.56	–	–	–	–	0.1	1	0.2	11	11.8	98
B3 (IgG1)	1.56	6.25	0.0063	0.025	–	–	–	–	–	–	0.2	6	0.3	12	24.7	98
118 (IgG3)	0.0063	0.0063	0.0063	0.025	1.56	6.25	58	9	2	5	NT		NT		NT	
F-12 (IgM)	–	–	0.39	0.39	12.5	25	–	–	–	–	0.1	1	0.2	4	1.2	54
Engineered 3S193																
Chimeric #5	0.0016	0.0125	0.025	25	0.39	0.39	–	–	–	–	0.4	43	0.8	47	46.2	99
Hybrid #6	0.0016	0.025	0.10	50	0.20	0.39	–	–	–	–	0.5	47	1.4	61	98.7	99
Humanized #7	0.0125	0.39	12.5	–	1.56	3.13	–	–	–	–	0.2	10	0.5	24	70.0	99
Humanized #11	0.0063	0.39	25	–	0.78	1.56	–	–	–	–	0.2	18	0.9	41	36.0	98

*Values are the minimum antibody concentrations (μg/ml) that show at least 50% rosette formation in rosetting assays or 50% lysis in cytotoxic tests. Each antibody was titrated at 2- or 4-fold serial dilutions from 100 μg/ml. –, No reactivity.

†For hemolysis test, the concentration of mAb was 70 μg/ml. –, Negative result. NT, not tested. PA-MHA, protein A-MHA; Rb α–mIg MHA, rabbit anti-mouse immunoglobulin-MHA.

‡MPC, mean positive count indicating fluorescence intensity. %+, percentage of positive cells. The concentration of mAb was 100 μg/ml. NT, not tested. Lymph., lymphocytes; Mono., monocytes; Granulo., granulocytes.

DISCUSSION

There are a number of reasons for selecting Le^y as an antigenic target for antibody-based therapeutic strategies in humans: high frequency of Le^y-expressing human tumors, homogenous Le^y expression in primary and metastatic lesions, and high density of Le^y determinants represented on the cell surface. In addition, Le^y antibodies of suitable isotypes mediate strong complement-dependent cytotoxicity and antibody-dependent cellular cytotoxicity. In fact, we have found that Le^y and Le^y-related structures appear to be the predominant cellular antigens eliciting cytotoxic antibodies in mice immunized with MCF-7 human breast cancer cells; cotyping initial hybridoma supernatants by cytotoxic tests and ELISA with synthetic Le^y indicated that 70% of the wells with cytotoxic antibody showed Le^y reactivity. A drawback of Le^y as an antigenic target in human cancers, shared with all tumor antigens identified to date, is the expression of Le^y in normal tissues. Epithelial cells in colon, stomach, breast, lung, and pancreas express Le^y, but certain cancers have been reported to express higher levels of Le^y than normal tissues (6–8). In addition to the degree of Le^y expression in normal and malignant tissues, the relative accessibility of normal vs. tumor tissue to circulating Le^y antibodies needs to be ascertained, information that will come from antibody biodistribution studies in patients.

Although it is well known that antibodies generated against peptides often do not react with the native protein, we had not expected this to be so with carbohydrate determinants such as Le^y. However, several key features distinguished antibodies raised against synthetic Le^y determinants and those raised against natural Le^y. (*i*) The anti-synthetic Le^y mAbs reacted well against synthetic Le^y determinants but poorly against natural Le^y; (*ii*) the isotypes of the antibodies raised against synthetic Le^y were IgG1, IgG2a, or IgG2b, in contrast to the IgM or IgG3 isotypes of antibodies to natural Le^y; and (*iii*) even the most specific of the anti-synthetic Le^y mAbs cross-reacted with Le^x or H-type 2 structures, whereas anti-natural Le^y mAbs could be isolated that showed apparent exclusive specificity for Le^y. These distinctions may be accounted for by differences in the density/concentration of Le^y determinants on the synthetic Le^y-neoglycoproteins as compared with natural Le^y products and by the influence of carrier protein and linker on the immunogenicity, conformation, and accessibility of Le^y epitopes. We have made attempts to modify the immune response to synthetic Le^y using different adjuvants and different immunization procedures, but these have not succeeded in changing specificity or mAb isotypes. Despite this inability of synthetic Le^y to generate antibodies that react efficiently against natural Le^y, Le^y and other synthetic oligosaccharide determinants are extremely useful in specificity testing of antibodies raised against natural carbohydrate determinants.

In addition to mAb 3S193 (the IgG3 anti-Le^y mAb discussed here), a number of other Le^y mAbs have been described (9–16). Two of the best characterized anti-Le^y mAbs are BR55-2 and B3. Both mAbs have high specificity for Le^y; however, they have been reported to react with closely related structures, such as BLe^y for mAb BR55-2 (12) and dimeric Le^x and extended Le^x in the case of mAb B3 (16). It will be interesting to determine the reactivity patterns of mAb 3S193 with these and other Le^y-related structures, such as trifucosylated Le^y and extended forms of Le^y (29). Knowledge of these cross-reactions is of more than academic interest, as illustrated by the hemolytic activity of mAb118 for O erythrocytes, an antibody originally thought to be highly specific for Le^y but subsequently found to have reactivity for the H-type 2 structure (14). To assess cross-reactivity with BGR determinants, a range of antibody con-

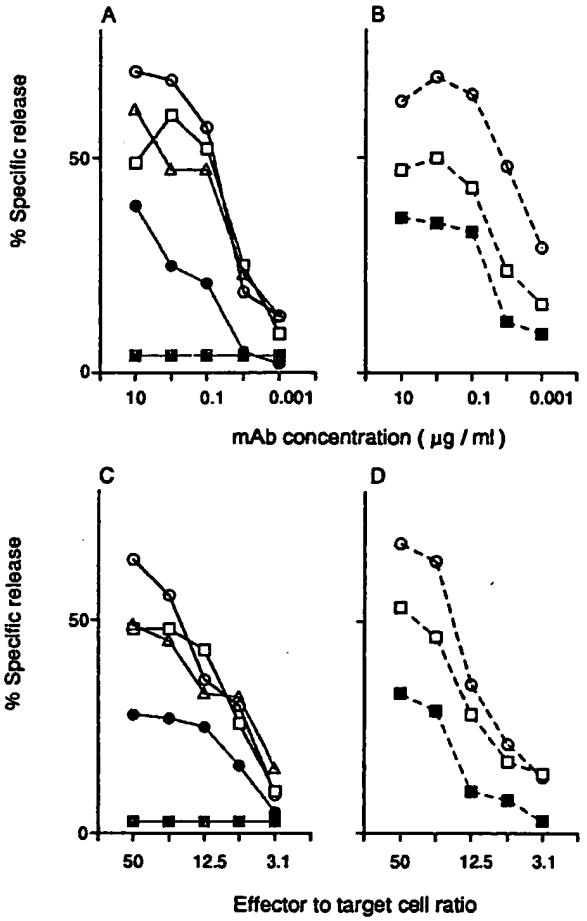

FIG. 1. ADCC mediated by humanized mAb 3S193#11. Five colon cancer cell lines (○, SW837; □, DLD-1; △, SW620; ●, SK-CO-10; ■, SW1222) (*A* and *C*) and three breast cancer cell lines (○, MCF-7; □, BT-20; ■, ZR-75-1) (*B* and *D*) were incubated with human lymphocytes and humanized mAb 3S193#11. *A* and *B* show percentage of specific release of ^{51}Cr at 10-fold serial dilutions of humanized mAb 3S193#11. The effector/target cell ratio was 50:1. Cytotoxicity by effector cells alone was 0–21%, depending on target cells, and these values are subtracted from the data given in each experiment. In *C* and *D*, humanized mAb 3S193#11 was tested at 1.0 μg/ml (*C*) or 0.1 μg/ml (*D*) at different effector/target cell ratios. Effector cells alone gave 0–23% cytotoxicity at each ratio, and data shown are obtained by subtracting these background values. Antibody alone gave ≤1% cytotoxicity.

centrations needs to be tested; cross-reactivity may not be observed at 10 μg/ml but it may be seen at 25 μg/ml, a level that will probably be exceeded in clinical trials of anti-Le^y antibodies. Further knowledge of the structures seen by Le^y reagents on cells should help explain the basis of the low reactivity of highly specific Le^y reagents for granulocytes in flow cytometry and the strong hemolysis of erythrocytes with mAb 8S202, a mAb that shows excellent Le^y specificity by all other tests.

Chimeric and humanized forms of mAb 3S193 were generated in an attempt to recreate the specificity of mouse mAb 3S193 in a form more acceptable for clinical use. Cross-reaction with Le^y-related antigens was seen for the chimeric antibody (3S193#5); this may be a consequence of the presence of nonauthentic residues at the termini of the variable regions derived from the expression vectors. Substitution of HuVK for the MuVK chain, giving mAb 3S193#6, exacerbated the cross-reactivity, whereas conversion to a fully humanized antibody (mAb 3S193#7) restored the specificity, suggesting that the nature of the heavy- and κ-chain variable-region interface influences antigen binding. Inclusion of an additional mouse framework residue in each chain produced a molecule, mAb 3S193#11, with improved reactivity and serological properties closely approximating those of its mouse counterpart. The availability of this humanized Le^y reagent with high specificity and strong biological functions will facilitate clinical exploration of Le^y as a therapeutic target in human cancer.

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Antibodies against GD2 Ganglioside Can Eradicate Syngeneic Cancer Micrometastases[1]

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ABSTRACT

After 10 years of clinical trials in patients with advanced cancer, monoclonal antibodies (mAbs) against cell surface antigens have not lived up to their initial promise. One such cell surface antigen is the ganglioside GD2. GD2 is richly expressed at the cell surfaces of human neuroblastomas, sarcomas, and melanomas. We have described a murine lymphoma (EL4) that is syngeneic in C57BL/6 mice and expresses GD2, a mAb against GD2 (mAb 3F8), and we have prepared a conjugate vaccine (GD2-keyhole limpet hemocyanin plus immunological adjuvant QS-21) that consistently induces antibodies against GD2. We demonstrate here, for the first time in a syngeneic murine model, that passively administered and vaccine-induced antiganglioside antibodies prevent outgrowth of micrometastases, and we use this model to establish some of the parameters of this protection. The level of protection was proportional to antibody titer. Treatment regimens resulting in the highest titer antibodies induced the most protection, and protection was demonstrated even when immunization was initiated after tumor challenge. Treatment with 3F8 1, 2, or 4 days after i.v. tumor challenge was highly protective, but waiting until 7 or 10 days after challenge resulted in minimal protection. The results were similar whether number of liver metastases or survival was used as the end point. These results suggest that unmodified mAbs or antibody-inducing vaccines against GD2 (and possibly other cancer cell surface antigens) should be used exclusively in the adjuvant setting, where circulating tumor cells and micrometastases are the primary targets.

INTRODUCTION

Most mAb[3] treatments have been performed on patients with advanced disease, and the treatments were of short duration, with response of measurable disease as the end point. Responses have been rare. Occasional regression of measurable neuroblastoma, melanoma, and breast cancer lesions and more frequent regression of B-cell lymphomas have resulted in patients treated with mAbs against cell surface antigens, including: gangliosides GM2[4] (1), GD2 (2–5), and GD3 (6–8); HER2 neu (9); and lymphoma idiotype antigens (10, 11). Trials with mAbs against GD2 are a case in point. The response rate in children with GD2-positive cancers (primarily neuroblastomas) treated with mAb 14.G2a or 3F8 is between 0 and 25% (12, 13), and in melanoma patients treated with mAb 3F8, 14.G2a, or chimeric 14.18, the response rate is between 0 and 22% (13, 14). A chimeric 14.18-interleukin 2 fusion protein shown to be potent in a *scid/scid* xenograft model (15) is now being considered for clinical trials. Neither immunogenic GD2 vaccines nor a syngeneic animal model has been previously available, making it difficult to compare these various approaches or to test the many variables associated with antibody-mediated therapies in the setting of a normal immune system.

As opposed to the minimal benefit seen with mAbs in patients with advanced disease, there is an expanding body of evidence indicating that antibodies can protect against subsequent tumor challenge in experimental animals and prevent tumor recurrence in humans. mAbs against several protein or glycoprotein tumor antigens have resulted in significant protection from syngeneic tumors in the mouse (16–19), mAb R24 against GD3 has resulted in protection from syngeneic melanoma growth in hamsters (20), and mAbs against GD2/GD3 (21) or GD2 (22) have resulted in protection against human tumor challenges in nude mice. There is also evidence in humans that natural antibodies, passively administered antibodies, or vaccine-induced antibodies against cancer antigens can result in prolonged disease-free and overall survival in the adjuvant setting. (*a*) Paraneoplastic syndromes have been associated with high titers of natural (not induced by vaccine or passive administration) antibodies against onconeural antigens expressed on neurones and certain malignant cells. The antibodies are apparently induced by tumor growth and have been associated with autoimmune neurological disorders and, in addition, with delayed tumor progression and prolonged survival (23–25). (*b*) Patients with American Joint Commission On Cancer stage III melanoma and natural antibodies against GM2 ganglioside studied at two different medical centers have an 80–90% 5-year survival, compared to the expected 40% rate (26, 27). (*c*) Patients with small cell lung cancer and natural antibodies against small cell lung cancer had prolonged survival, compared to antibody-negative patients (28). (*d*) Patients with Dukes' C colon cancer treated with mAb 17-1A in the adjuvant setting had a significantly prolonged disease-free and overall survival, compared to randomized controls (29). (*e*) Antibody responses induced by vaccines in the adjuvant setting have been correlated with subsequent prolonged disease-free and overall survival (26, 27, 30–33).

Given the potential clinical importance of a variety of cell surface antigens, including ganglioside GD2, as targets for mAbs and cancer vaccines inducing an antibody response, we have identified a suitable syngeneic mouse model to address some of the variables associated with antibody-mediated protection from and therapy of cancer. EL4 is a lymphoma syngeneic in C57BL/6 mice that we have previously reported to express GD2 (34). It is a unique model, in that GD2 is also a human tumor antigen, against which there is not only a clinically active mAb but also a consistently immunogenic conjugate vaccine, GD2-KLH plus QS21. We demonstrate here that passively administered and vaccine-induced antibodies are able to prevent establishment of subsequently administered EL4 challenge and to eliminate EL4 micrometastases when administered after EL4 challenge, and we define some of the parameters of this protection.

MATERIALS AND METHODS

mAbs and Vaccine

The origins of mAb 3F8 (IgG3) against GD2 (35), mAb 696 (IgM) against GM2 (36), mAb 013 against a primitive human neuroectodermal bone tumor (37), and mAb IE3 against Tn antigen (38) have been described. Neither O13

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[3] The abbreviations used are: mAb, monoclonal antibody; KLH, keyhole limpet hemocyanin; CDC, complement-dependent cytotoxicity.

[4] The designations GM2, GD2, and GD3 are used in accordance with the abbreviated ganglioside nomenclature of Svennerholm (48).

Table 1 *Experiment 1: liver metastases after i.v. challenge with EL4 lymphoma incubated with mAbs 3F8 (against GD2) and 696 (against GM2)*[a]

mAb	No. of mice	No. of tumors in liver	Liver mass (g)
PBS (control)	8	57.8 ± 67.2	2.59 ± 1.03
mAb 696	5	94 ± 100	2.52 ± 1.21
mAb 3F8	5	0	1.23 ± 0.04[b]
mAb 696 + mAb 3F8	5	0	1.20 ± 0.14[b]

[a] After incubation with 100 μg/ml 3F8 and 50 μg/ml 696 for 1 h, 3×10^4 EL-4 cells per mouse were injected (i.v.) into C57BL/6 mice. Thirty-four days after challenge, mice were sacrificed, and the livers were evaluated. Results are expressed as mean ± SD.
[b] $P < 0.01$, compared with PBS control group.

nor IE3 reacts with EL4. Immunological Adjuvant QS-21, a purified saponin fraction (39), was obtained from Aquilla Biopharmaceuticals Inc. (Worcester, MA). GD2 and GD2 conjugated to KLH were provided by Progenics Pharmaceuticals Inc. (Tarrytown, NY). Conjugation of GD2 to KLH was achieved by conversion of the GD2 ceramide double bond to aldehyde by ozonolysis and attachment to KLH by reductive amination in the presence of cyanoborohydride, as described previously for GD3 (40). Each GD2-KLH vaccine contained 10 μg of GD2 conjugated to 60 μg of KLH, plus 10 μg QS-21. Vaccines were administered s.c. three times at 1-week intervals, except in the final experiment, when they were administered at 4-day intervals.

Mice and Cell Lines

C57BL/6 mice (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The EL4 cell line was established from lymphoma induced in a C57BL/6 mouse by 9,10-dimethyl-1,2-benzanthracene. It has recently been shown to express GD2 ganglioside (34). EL4 was maintained in 10% FCS-RPMI. For tumor cell challenges, EL4 cells were washed three times in PBS, and 3×10^4 cells (in the final experiment, 5×10^3cells) were injected i.v. into the tail vein. At the indicated time points, mice were sacrificed, and livers were removed, weighed, and fixed in 10% formalin. Metastases were also frequently present in lymph nodes and other sites (although rarely in the lungs), but hepatic metastases were easiest to quantitate. Hepatic metastases were detected as white nodules on the liver surface.

Serological Assays

ELISA. ELISAs were performed as described previously (41). GD2 or GM2 in ethanol was coated on ELISA plates at 0.1 μg/well. A series of antiserum dilutions were incubated with the coated ganglioside for 1 h. Secondary antibodies were alkaline phosphatase-conjugated goat antimouse IgG or IgM at a dilution of 1:200 (Southern Biotechnology Associates, Inc., Birmingham, AL). ELISA titer is defined as the highest dilution yielding an absorbance of 0.1 or greater over that of normal control mouse sera. mAbs 3F8 and 696 were used as positive controls in each assay.

Flow Cytometry. EL4 cells (3×10^5) were incubated with 40 μl of 1:30 diluted antisera or 1:2 diluted mAb supernatant for 30 min on ice. After washing with 3% FCS in PBS, the cells were incubated with 20 μl of 1:15 diluted FITC-labeled goat antimouse IgG or IgM (Southern Biotechnology Associates, Inc.). The positive population of the stained cells was quantitated by flow cytometry (EPICS-Profile II; Coulter Co., Hialeah, FL), as described previously (41).

CDC. In 100 μl of 5% FCS in RPMI, 2×10^5 EL4 cells were incubated with 10 μl of 1:10 mouse antiserum or 10 μg/ml mAb for 10 min. Thirty μl of complement (guinea pig; Sigma Chemical Co.) were added and incubated at 37°C for 4 h. Thirty μl of 0.4% trypan blue were added, and after 3 min, dead and viable cells were counted (41).

Statistical Methods

Experimental groups were compared to controls for number of hepatic metastases, survival, or antibody titers using the Mann-Whitney two-sample *t* test (42).

RESULTS

Having previously shown that mAb 3F8 was able to bind to EL4 and induce potent CDC and antibody-dependent cell-mediated cytotoxicity (3, 13, 35), we performed a series of experiments progressively testing the ability of passively administered and then actively induced antibodies against GD2 to eradicate hepatic micrometastases (experiments 1 and 2) and to prolong survival (experiments 3–7).

Effect of mAb Administration on Hepatic Metastases (Experiments 1 and 2). In experiment 1, we mixed 3F8 or negative control mAb 696 with the EL4 lymphoma cells prior to challenge to confirm *in vivo* impact of antibody binding. EL4 cells were incubated for 1 h with PBS, mAb 696 (against GM2, which is minimally expressed on EL4), mAb 3F8, or mAbs 696 and 3F8 prior to i.v. challenge. All mice were sacrificed on day 34, hepatic metastases were counted, and livers were weighed (Table 1). Only EL4 preincubation with 3F8 ± 696 eliminated metastases. In experiment 2, mice were injected i.v. with PBS, negative control antibody IE3 (100 μg), or one of three doses of 3F8 (50, 100, or 250 μg) 2 h before i.v. challenge with untreated EL4 cells. Mice were sacrificed at day 30. Administration of all three doses of 3F8 eliminated metastases in most mice (Table 2).

Effect of mAb Administration or Vaccination on Survival (Experiments 3–6). In experiment 3, two groups of six mice received a single i.v. injection of 200 μg of 3F8 1 day before or 2 days after EL4 i.v. challenge. Three additional groups of six mice were vaccinated three times (on days −21, −14, and −7) prior to EL4 challenge. They were vaccinated with PBS, 10 μg of GD2 mixed with 60 μg of KLH plus QS21 (negative controls), or 10 μg of GD2 conjugated to 60 μg of KLH plus QS21. Mice receiving the conjugate vaccine survived significantly longer than did the control mice ($P < 0.008$), and one mouse was sacrificed on day 100 with no evidence of tumor. Five of six mice receiving 3F8 1 day before challenge and five of six mice receiving 3F8 2 days after challenge also remained tumor free (Fig. 1, *Experiment 3*). All negative control mice died by day 28.

Experiments 4 and 5 focused on treatment with mAb. In experiment 4, groups of four or five mice received PBS or 3F8 2 days or 4 days after EL4 challenge i.v. All 3F8-treated mice survived longer than did control mice ($P < 0.004$), and three mice in the 3F8 groups remained tumor free (Fig. 1, *Experiment 4*). Experiment 5 compared treatment with PBS or mAb O13 (negative controls) and treatment with 50 or 200 μg of 3F8, all administered 2 days after EL4 challenge i.v. (Fig. 1, *Experiment 5*). Once again, all 3F8-treated mice survived longer than did any control mouse ($P < 0.004$), and most mice (8 of 12) treated with either dose of 3F8 remained tumor free.

Experiment 6 again compared immunization prior to tumor challenge with mAb treatment at various intervals after challenge. All vaccinated mice again survived longer than did any control mouse ($P < 0.004$), and four of six mice remained disease free (Fig. 1, *Experiment 6b*). Most mice receiving 70 μg of 3F8 2 or 4 days after challenge remained disease free. However, the same dose 7 or 10 days after challenge had no significant effect (Fig. 1, *Experiment 6a*). Experiment 6 was a single experiment but is presented in two panels for greater clarity. Once again, the relevant negative control treatments (mAb R24 against GD3, which is not expressed on EL4, and

Table 2 *Experiment 2: liver metastases after i.v. injection of mAbs followed by i.v. EL4 challenge*[a]

Treatment	No. of mice	No. tumors in liver	Liver mass (g)
PBS (control)	7	29.2 ± 14.8	1.90 ± 0.47
mAb IE3 (100 μg/mouse)	9	17.6 ± 15.9	1.95 ± 0.72
mAb 3F8 (50 μg/mouse)	6	0[b]	1.03 ± 0.13
mAb 3F8 (100 μg/mouse)	6	4.3 ± 7.0[c]	1.17 ± 0.41
mAb 3F8 (250 μg/mouse)	6	0[d]	0.90 ± 0.16

[a] Challenge was with 3×10^4 EL-4 cells 2 h after mAb injection. The mice were sacrificed 30 days after challenge, and the livers were evaluated. Results are expressed as mean ± SE.
[b] $P < 0.01$, compared with PBS control group.
[c] $P < 0.02$, compared with PBS control group.
[d] $P < 0.001$, compared with PBS control group.

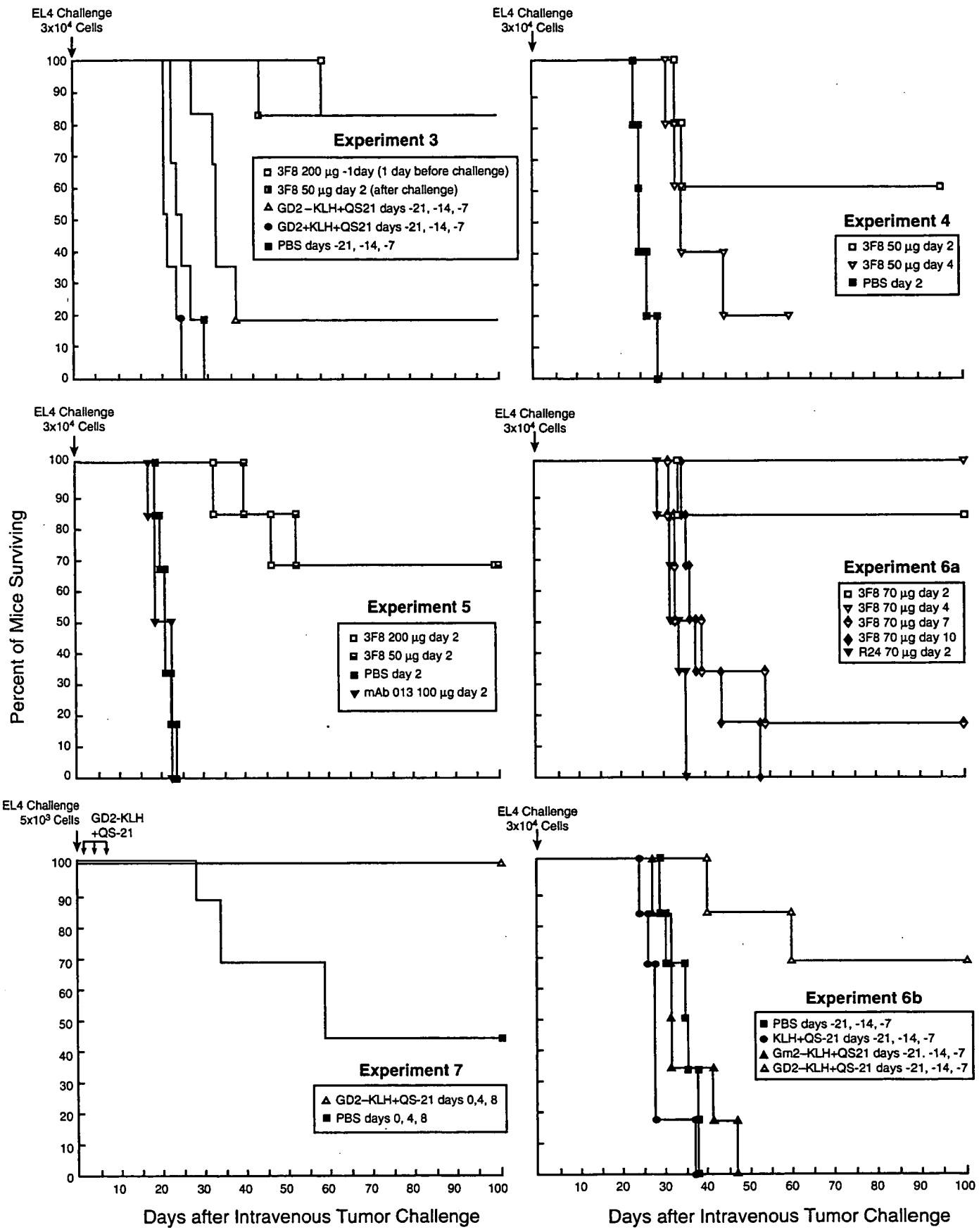

Fig. 1. Survival of groups of four to six mice treated in five separate experiments with 3F8 mAb, GD2-KLH plus QS-21 vaccine, and various control treatments, after i.v. challenge with syngeneic EL4 lymphoma cells. 3F8 mAb against GD2 administered prior to challenge or 1–4 days after challenge and GM2-KLH plus QS-21 vaccination prior to challenge or starting immediately after challenge were both protective.

Table 3 *Antibody reactivity in sera of mice treated with GD2-KLH vaccine or mAb 3F8*[a]

Treatment	No. of mice	Reciprocal ELISA titer		Flow cytometry (% positive cells)		CDC (% dead cells)
		IgM	IgG	IgM	IgG	
Experiment 3						
PBS (100 μl)	6	0/0	0/0	3.1–3.3/3.2	3.1–3.4/3.2	10–10/10
GD2 + KLH + QS21 (10 μg + 60 μg + 10 μg)	6	0–80/40	0	5.0–8.9/6.5	4.2–5.5/4.5	Not tested
GD2-KLH + QS21	6	40–640/80	80–2,560/640	18–88/57	22–93.6/60.5	20–60/40
3F8 (250 μg), 1 day before challenge	6	0/0	2,560–5,120/5,120	Not tested	99–99.3/99	90–95/95
Experiment 6						
PBS (100 μl)	6	0/0	0/0	1.5–2.9/2.0	1.4–2.6/1.7	10–15/10
KLH + QS21 (60 μg + 10 μg)	6	0/0	0/0	1.3–8.1/3.6	1.3–2.2/2.0	10–15/10
GD2-KLH + QS21	6	160–640/320	180–14,580/1,620	57–99/95	13–99/89	40–80/60
3F8 (250 μg)	6	0/0	1,620–4,860/3,240	Not tested	98–100/99	85–95/95

[a] Mice were bled 7 days after the third immunization with GD2 vaccine or 4–5 days after mAb 3F8 injection. Results are expressed as range/median.

vaccination with KLH plus QS21, GM2-KLH plus QS21, and PBS, which do not induce anti-GD2 antibodies) had no effect.

Correlation between Serum Antibody Titer and Survival. Serum anti-GD2 antibody titers immediately after 3F8 administration were not tested, but they ranged between 1:1620 and 1:4860 (median, 1:4860) 3–5 days later, except in experiment 4, in which they were between 1:180 and 1:4860 (median, 1:540). Vaccine-induced antibody titers ranged between 1:640 and 1:1620 for IgG and 1:80 and 1:1620 for IgM (Table 3). Comparable antibody titers by ELISA resulted in comparable reactivity by flow cytometry and complement-mediated cytotoxicity, whether due to 3F8 or vaccine administration. In both cases, protection from subsequent tumor challenge resulted. A correlation between antibody titer and *in vivo* protection is suggested by these results. Administration of 3F8 resulted in higher serum titers against GD2 than vaccine administration in both experiments ($P < 0.004$ for CDC) and greater protection ($P < 0.008$ for experiment 3). In experiment 4, in which 3F8 levels were lower than expected after 3F8 administration, survival was lower as well. Vaccine-induced antibody titers prior to challenge were higher in experiment 6 than in experiment 3, and protection was greater as well ($P < 0.025$).

Therapeutic Vaccination. Because 3F8 administration 7 or 10 days after EL4 challenge with 3×10^4 resulted in minimal protection, this suggested that vaccination after challenge, which was normally performed at weekly intervals and required 14–21 days for antibody induction, would be ineffectual. Consequently, we performed one final experiment aimed at testing the ability of vaccinations started after tumor challenge to prolong survival. In experiment 7, the number of EL4 cells per challenge was decreased from 3×10^4 to 5×10^3 cells, and the vaccines were administered on days 0, 3, and 7, beginning immediately after the challenge. Median IgM and IgG antibody titers on days 13 and 18 were both 1:320. Protection was again seen (Fig. 1, *Experiment 7*), although the difference was not statistically significant ($P = 0.15$).

DISCUSSION

The mechanism of antibody effect against bacteria is predominantly complement mediated inflammation and cytotoxicity (CDC; Ref. 43). Although other effector mechanisms have been suggested for GD2 antibody, such as inhibition of tumor cell substratum or extracellular matrix interactions (22), activation of immune effector mechanisms remains the most likely explanation. 3F8, the anti-GD2 mAb used here, is an IgG3 antibody that is particularly potent at inducing complement-mediated inflammation/cytotoxicity and antibody-dependent cell-mediated cytotoxicity. We have previously demonstrated, in melanoma patients, that natural or vaccine-induced IgM antibodies against GM2 ganglioside correlated with improved disease-free and overall survival (26, 44) and that a GM2-KLH plus QS21 vaccine induced IgM and IgG antibodies in melanoma patients, which were both able to mediate CDC (45). Fortuitously, the IgG subclasses were IgG1 and IgG3 (44–46), the two human subclasses best able to mediate CDC. The same applies to the murine model we describe here. IgM and IgG antibodies were induced in all vaccinated mice, these antibodies and administered 3F8 mAbs were able to mediate potent CDC, and antibody titers correlated with survival and inversely with the number of hepatic metastases. Although mAbs administered up until 4 days after challenge were able to completely prevent tumor growth in most mice, by 7–10 days after challenge, 3F8 administration had little effect. This strongly suggests that treatment with mAbs or vaccines inducing antibodies must be restricted to the adjuvant setting, where the targets are circulating tumor cells and micrometastases, and it may explain why mAb treatment trials in patients with measurable tumor burdens have not been more successful.

Passively administered and vaccine-induced antibodies were both able to protect against growth of micrometastases. There are advantages and disadvantages to each approach. Therapy in the adjuvant setting may require repeated treatments to maintain antibody titers over a prolonged period to overcome the issue of tumor cell dormancy and sanctuary sites. Except in immunosuppressed patients, this excludes murine mAbs, which would be eliminated within weeks by human antimouse antibodies. Chimeric, humanized, or human mAbs would overcome this issue but would be subject to elimination by anti-idiotypic antibodies. On the other hand, in the absence of human antimouse antibodies or anti-idiotypic antibodies, higher serum antibody levels than could be induced by vaccination are assured after mAb administration, and such antibodies have been or could be produced against most antigens. Vaccines against most defined tumor antigens are more practical to produce and administer because they can be administered s.c. and at longer intervals. Phase III trials with GM2-KLH and sialyl Tn-KLH vaccines that consistently induce moderate titers of antibodies against these antigens are currently ongoing in the adjuvant setting in patients with melanoma and breast cancer (33, 45). Because the antibody response seems to be polyclonal, antibody inactivation by anti-idiotypic antibodies has not been a problem and specific antibody levels have been maintained against GM2 by immunizations at 3- or 4-month intervals for over 2 years (45). However, even the most potent conjugate vaccines have not been able to induce consistent antibody responses against all antigens, and the titers are never as high as can be achieved with mAb administration. The results obtained here, demonstrating the ability of either approach to protect against tumor challenge and to eliminate micrometastases, in the absence of any detectable toxicity, argue strongly in favor of the careful use of either approach or the combination.

GM2-KLH and GD2-KLH have both proven consistently immunogenic and safe in melanoma patients, whereas GD3 (the major melanoma ganglioside)-KLH has not proven so immunogenic (reviewed in Ref. 47). Adjuvant therapy of melanoma might optimally include a bivalent conjugate vaccine (GM2-KLH plus GD2-KLH), a humanized anti-GD3 mAb, or a combination of bivalent vaccine plus mAb.

Vaccines against infectious diseases do not prevent infection; they limit its spread from its point of contact. Postcontact boosts in antibody titers, even in protected hosts, attest to active infection at the contact site. This is most striking when time has elapsed because the original infection and antibody titers have fallen to low levels but rise to protective levels within 4–7 days, preventing symptomatic infection. In patients with cancer, we see the adjuvant setting (after removal of the primary cancer or positive lymph nodes) as being quite similar to the picture in patients being reexposed to infectious diseases. The primary targets in both cases are circulating pathogens and microscopic spread, and in the case of infectious diseases, antibodies are the primary method of protection. We demonstrate here, with passively administered mAbs and vaccine-induced antibodies against the defined cancer antigen GD2 ganglioside, that antibodies can also protect mice against circulating syngeneic tumor cells and micrometastases. If antibodies of sufficient titer and potency to eliminate circulating cancer cells and micrometastases could be maintained in cancer patients as well, even metastatic cancer would have quite a different implication. With continuing showers of metastases no longer possible, aggressive treatment of primary and metastatic sites might result in long-term control.

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